

ZERO-FIELD SPLITTING OF Fe^{3+} IN HORSERADISH PEROXIDASE AND OF Fe^{4+} IN HORSERADISH PEROXIDASE COMPOUND I FROM ELECTRON SPIN RELAXATION DATA

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ABSTRACT From the temperature dependence of the Orbach relaxation rate of the paramagnetic center in horseradish peroxidase (HRP), we deduce an excited-state energy of 40.9 ± 1.1 K. Similar studies on the broad EPR signal of HRP compound I indicate a much weaker Orbach relaxation process involving an excited state at 36.8 ± 2.5 K. The strength of the Orbach process in HRP-I is weaker than one would normally estimate by 2–4 orders of magnitude. This fact lends support to the model of HRP-I involving a spin $1/2$ free radical coupled to a spin 1 Fe^{4+} heme iron via a weak exchange interaction. Such a system should exhibit an Orbach relaxation process involving ΔE , the excited state of the Fe^{4+} ion, but reduced in strength by $(J_{yy}/\Delta E)^2$, where J_{yy} is related to the strength of the exchange interaction between the two spin systems.

The enzymatic reaction cycle of horseradish peroxidase (HRP) yields two intermediate complexes (1): HRP compound I (HRP-I) and HRP-II. They are known to be two and one oxidation states above the native ferric enzyme, respectively. Much speculation about the electronic state of the active centers in these intermediate complexes has occurred (2–8). We present here our electron spin-lattice relaxation data on both HRP and HRP-I. From these data we are able to measure the first excited electronic states of iron in both of these proteins. In addition, by comparing the strengths of the Orbach relaxation rate in these two systems, we are able to confirm the weak nature of the exchange coupling between the Fe^{4+} ($S = 1$) ion and the free radical in HRP-I.

HRP isozyme C was purified by the method of Shannon et al. (9). All enzyme samples used in these experiments had an R_z -value (A_{403}/A_{280}) of at least 3.35. The concentration of HRP solutions was determined on the basis of a molar absorptivity of 1.02×10^5 at 403 nm. Purified HRP was dialyzed against 0.05 M potassium phosphate buffer (pH = 6.5). Following dialysis these HRP samples were adjusted to a concentration of ~ 5 mM in 40% glycerol (vol/vol). Benzohydroxamic acid was then added to the HRP samples to give a final molar ratio of benzohydroxamic acid to HRP of 1.2:1.

To prepare HRP-I, purified HRP preparations were dialyzed against 0.05 M potassium phosphate buffer (pH = 6.5) and adjusted to a concentration of ~ 2 mM. Peracetic acid was added to yield a molar ratio of peracetic acid to enzyme of 1.5:1. The purity of HRP-I samples was

checked by visible spectral measurements. All preparations were found to contain at least 95% HRP-I. These preparations were frozen in liquid N_2 immediately after their synthesis.

Because the Orbach relaxation process (10) involves a real transition from a ground energy spin state to an excited electronic state, ΔE above ground, a unique temperature dependence involving ΔE is observed. Scholes et al. (11) were the first to apply this probe to high-spin ferric proteins to determine the zero field splitting of the $S = 5/2$ sextet. The technique has since become standard practice (12–14).

Spin-lattice relaxation rates, $1/T_1$, were measured using the pulse saturation and recovery technique with a custom-built superheterodyne x -band spectrometer incorporating 50-dB microwave diode switches and a 60 MHz intermediate frequency amplifier with a 10 MHz band width. Of particular importance in the evaluation of our results is our method of temperature measurement and control. A calibrated germanium resistance thermometer was thermally linked to the microwave cavity, which was also wrapped with a 75-cm, 500- Ω heater wire. The thermometer, heater, and cavity were thermally isolated from the He-4 bath by a surrounding copper can containing He-4 exchange gas. Microwave power was fed through the isolation can and into the cavity via thin-walled stainless steel waveguide and a Gordon coupler (15). Located at the bottom of the isolation can was an access port sealed with an indium o-ring. The germanium resistance thermometer formed one arm of an AC resistance bridge. Any bridge unbalance

was phase sensitively detected and used to control power to the heater. In this manner temperatures between 1.4 and 25 K could be controlled to within a few millidegrees Kelvin.

After preparation, protein samples were placed in direct thermal contact with the removable lower half of the copper microwave cavity and frozen at liquid N₂ temperature. Thin, vertical teflon baffles confined the samples to regions of large microwave magnetic fields to insure a uniform saturation of the EPR signal throughout the sample volume. It was possible to install the bottom half of the microwave cavity (with its frozen sample) onto the precooled upper half-cavity through the access port and to seal the port with an indium o-ring before the sample warmed excessively. After sample loading, the isolation can was flushed with dry He-4 gas to purge all N₂ vapor. The can was then immersed directly into liquid N₂.

Our sample of HRP exhibited a clean EPR absorption signal, with no extraneous resonances in evidence. We have estimated the three principal *g*-values using an absorption derivative spectrum from the frozen solution. Magnetic field values corresponding to a low field derivative maximum, midfield derivative zero, and a high field derivative minimum yield *g*-values of 6.14, 5.45, and 2.03 for this high spin ferric protein. These values suggest a substantial (~32% amplitude) spin admixture of the *S* = 3/2 manifold into the ground state wave function since the average of the two higher *g*-values falls significantly below 6. Our *g*-values are not inconsistent with those reported by Leigh et al. (16).

Fig. 1 shows the fit of our relaxation data on HRP to the sum of direct and Orbach relaxation rates. The Orbach process is characterized by an excited state energy of 40.9

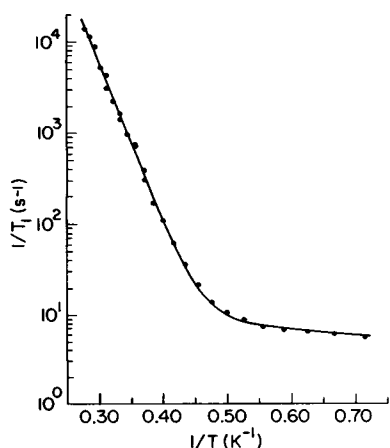


FIGURE 1 Spin-lattice relaxation rates of native HRP between temperatures of 1.4 and 3.6 K at a microwave frequency of 9.456 GHz. The higher temperature data indicate a dominant Orbach relaxation mechanism involving an excited-state energy of 40.9 ± 1.1 K above the ground doublet of this high spin ferric protein. The microwave frequency and magnetic field correspond to a *g*-value of 5.81. $\nu_0 = 9.456$ GHz. $H = 1,162$ G. $1/T_1 = AT + B/(e^{\Delta/T} - 1)$. $A = 4.14 \pm 0.34$ s⁻¹K⁻¹. $B = (1.26 \pm 0.50) \times 10^9$ s⁻¹.

± 1.1 K and a prefactor of 1.26×10^9 s⁻¹. Prefactors of $10^8 - 10^9$ s⁻¹ are typical for Orbach processes in high spin ferric proteins (11) and in rare earth salts (17) provided that the excited state splitting is reasonably large (>30 K). The relaxation rate between 1.4 and 3.7 K is given by

$$1/T_1 = 4.14T + 1.26 \times 10^9 (e^{40.9/T} - 1)^{-1} \text{ s}^{-1}, \quad (1)$$

where *T* is the absolute temperature.

This value of ΔE is 25% larger than that reported in an abstract by Scholes et al. (18). They found ΔE to be 32.5 ± 1.1 K for HRP in a 50% glycerol-water frozen solution of electrophoretically purified HRP. No further purification was made (personal communication, C. P. Scholes). Their measurements were taken from $1/T = 0.75$ K⁻¹ to $1/T = 0.27$ K⁻¹, where the relaxation rate was observed to be $\sim 10^4$ s⁻¹. At low temperatures they measured the rate to vary approximately as $T^{2.4}$. Differences in sample and isozyme purity may account for these different dependences.

Our sample of HRP-I showed an EPR spectrum which agreed well with that reported in the literature (8, 19). Shown in Fig. 2 are the relaxation data on HRP-I which we took in the temperature range from 1.5 to 10 K. Fig. 2 differs from Fig. 1 in two important aspects: (a) the low temperature relaxation data on HRP-I fall somewhere between a *T* dependence (upper curve in the lowest temperature region) and a *T*² dependence (lower curve in the lowest temperature region); and (b) the strength of the

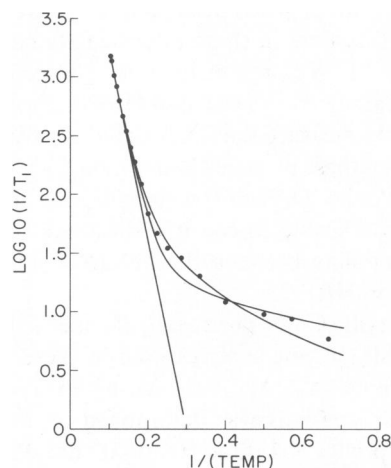


FIGURE 2 Spin-lattice relaxation rates of horseradish peroxidase compound I taken over the temperature interval between 1.5 and 10 K. The units of temperature (TEMP) and *T*₁ are Kelvin and seconds, respectively. $1/T_1 = AT^n + B/(e^{\Delta/T} - 1)$. $\nu_0 = 9.425$ GHz. $H = 3,450$ G. From the analysis discussed in the text, we deduce the presence of an Orbach relaxation mechanism that is forbidden in lowest order of perturbation theory and involves an excited state of the Fe⁴⁺ (*S* = 1) heme iron at an energy of 36.8 ± 2.5 K. The temperature dependence of this Orbach rate is indicated by the straight line in the figure. The upper and lower curves at the lowest temperatures include *T* and *T*² dependencies, respectively, and indicate that the low-temperature data fall between these two power laws. The microwave frequency and magnetic field correspond to a *g*-value of 1.95.

Orbach relaxation rate in HRP-I is smaller than in HRP by a factor of nearly 2×10^4 .

In arriving at an excited state energy of 36.8 ± 2.5 K we have taken the high temperature limit of three different fitting curves of the general form:

$$1/T_1 = A T^n + B(e^{\Delta/T} - 1)^{-1}, \quad (2)$$

where $n = 1$, $n = 2$, and the best fitting value, $n = 1.58$. This latter value lacks any theoretical justification, and serves only as an aid in extracting a reliable value of Δ from the higher temperature data. As more and more low temperature data were ignored, these three fitting curves converged to a common value of $\Delta = \Delta E/k = 36.8 \pm 2.5$ K. Subject to the constraint that Δ equals 36.8 K, the best fit of the data in Fig. 2 to Eq. 2 results from $n = 1.58 \pm 0.08$, $A = 3.23 \pm 0.26 \text{ s}^{-1} \text{ K}^{-1.58}$, and $B = (5.57 \pm 0.20) \times 10^4 \text{ s}^{-1}$. Our determination of Δ differs from that reported by Schulz et al. (8). On the basis of continuous wave saturation measurements at four temperatures, they reported an Orbach relaxation rate with an excited state energy of 29 ± 4 K. They used EPR measurements to establish relative temperatures and one thermometer reading to establish the absolute scale. Because their thermometer read the temperature upstream from the sample in a cold gas flow system, they apparently underestimated their sample temperature by $\sim 20\%$.

Finally, our observation of a relatively weak Orbach relaxation process in HRP-I further supports the model (6, 8) of a free radical ($S' = 1/2$) exchange coupled to an Fe^{4+} ($S = 1$) state of the heme iron according to the Hamiltonian:

$$\mathcal{H} = D(S_z^2 - 2/3) + E(S_x^2 - S_y^2) + \mu_B \vec{H} \cdot (\vec{g} \cdot \vec{S} + \vec{g}' \cdot \vec{S}') - \vec{S} \cdot \vec{J} \cdot \vec{S}'. \quad (3)$$

Here $S = 1$, $S' = 1/2$, μ_B is the Bohr magneton, and D and E represent the axial and rhombic crystal field parameters, respectively, of the Fe^{4+} ion. With E equal to zero, the Fe^{4+} ion is split into a lowest singlet ($S_z = 0$) and an upper doublet ($S_z = \pm 1$) at an energy D above the singlet. The inclusion of a free radical doubles the degeneracy of each level, but in the absence of any exchange coupling between the triplet and doublet states, an Orbach relaxation process of the doublet states is strictly forbidden. Such a process is allowed only when the exchange interaction $\vec{S} \cdot \vec{J} \cdot \vec{S}'$ produces a first-order mixing of the wave functions from the $S = 1$ and $S' = 1/2$ manifolds. The relaxation ($1/T_1$) and line-broadening ($1/T_2$) mechanisms of the Orbach relaxation process are related (20) by

$$\frac{1}{T_1} \propto \frac{B_1 B_2}{(B_1 + B_2)} (e^{\Delta/T} - 1)^{-1}, \quad (4a)$$

and

$$\frac{1}{T_2} \propto (B_1 + B_2) (e^{\Delta/T} - 1)^{-1}, \quad (4b)$$

where B_1 and B_2 are the squares of appropriate matrix

elements between members of the ground doublet and one of the excited states. Because the dynamic orbit-lattice Hamiltonian acts only on the $S = 1$ states, orthogonality of the zero-order wave functions will ensure that either B_1 or B_2 is zero in the absence of wave function admixtures from the exchange interaction. Based upon the weak exchange interaction ($J_{xx}S_xS'_x + J_{yy}S_yS'_y + J_{zz}S_zS'_z$) obtained by Schulz et al. (8), the amount of wave function admixture will be on the order of $J_{yy}/D \sim 2/36.8$ and the strength of the Orbach relaxation rate in HRP-I should be weaker than normal by a factor of ~ 340 . Our relaxation data indicate a difference of 2.2×10^4 in the prefactors of the Orbach relaxation rates in HRP and HRP-I. It is reasonable to assume that the relaxation rates of Fe^{3+} and Fe^{4+} could differ by a factor of 65 and account for this additional factor between the two observed relaxation rates.

It should also be noted from Eq. 4b that the line width broadening ($1/T_2$) in HRP-I due to the Orbach process will not be so reduced. This is an example of a system for which the Orbach relaxation rate has an extremely large T_1/T_2 ratio.

The details of the relaxation mechanism in HRP-I are quite similar to those reported by Calvo et al. (14) for the reduced primary quinone in reaction centers from *Rhodospseudomonas sphaeroides*.

Spin relaxation rates have been measured for the hydrogen peroxide compound of cytochrome *c* (21) using the two techniques of continuous wave saturation and phase shift detection under conditions of high frequency, fast passage modulation. The measured rates could be interpreted as indicative of an Orbach relaxation rate with a slightly anisotropic value of Δ ranging between 25 and 28 K, with prefactors corresponding to $2.6 \times 10^7 \text{ s}^{-1}$ and $2.0 \times 10^8 \text{ s}^{-1}$, respectively. Although this compound is thought to differ substantially from HRP-I, the basis for this faster Orbach relaxation process is not obvious. It is worth noting, however, that our measurements represent a final recovery rate (near thermal equilibrium) for a signal which was not strictly exponential over the full recovery range. Relaxation rates for the H_2O_2 compound of cytochrome *c* were measured using nontransient techniques that could have biased the results in the other direction, i.e., toward the initial recovery rates. In either case, it is only the temperature dependence of the relaxation rate that determines the quantity of interest, the excited state energy.

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